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Characterization of the Recombinant Human Receptor for *Escherichia coli* Heat-stable Enterotoxin*

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We report here the molecular characterization of a recombinant cell line (293-STaR) expressing the heat-stable enterotoxin receptor (STaR) from human intestine. We have compared the 293-STaR cell line with the human colonic cell line T84 that endogenously expresses STa binding sites. Scatchard analysis of displacement binding studies revealed a single STa binding site with an affinity (K_i) of 97 pM in 293-STaR compared with 55 pM in T84 cells. Saturation isotherms of STa binding gave a K_d of 94 pM for the cloned receptor expressed in 293 cells and 166 pM for the receptor present in T84 cells. Kinetic measurements of STa binding to 293-STaR gave an association rate constant, K_1, of 2.4×10^8 M^{-1} min^{-1} and a dissociation rate constant, K_2, of 0.016 min^{-1}. The half-time of dissociation was 43 min, and the K_d calculated from the ratio of the kinetic constants was 67 pM. The pH profile of STa binding showed that the number of STa binding sites is increased 3-fold at pH 4.0 compared with pH 7.0, with no effect on binding affinity. A polyclonal antibody directed against the extracellular domain of STaR immunoprecipitated two proteins of approximately 140 and 160 kDa from both 293-STaR and T84 cells. Cross-linking of ^{125}I-STa to 293-STaR cells resulted in the labeling of proteins with a molecular mass of approximately 153, 133, 81, 68, 56, and 49 kDa, the two smallest being the more abundant. Similar results have been reported for the STaR present on rat brush border membranes. These data suggest that the STaR-guanylyl cyclase identified by molecular cloning is the only receptor for STa present in T84 cells.

Escherichia coli heat-stable enterotoxins (STa) are small peptides of 18 or 19 amino acids known to cause diarrhea (1, 2). The toxins bind to specific cell surface receptors located on the intestinal brush border (3, 4) and activate guanylyl cyclase which results in an increase in the intracellular cGMP content of the cell (5–7). This increase in turn inhibits sodium chloride absorption and stimulates chloride secretion (5, 8, 9). This imbalance of ions is accompanied by a massive accumulation of water in the gut that gives rise to the diarrhea and dehydration that characterize enterotoxin activity.

It has been suggested that the STa binding site and the guanylyl cyclase activity are on separate proteins (10, 11). However, membrane-bound receptor-guanylyl cyclases have recently been cloned from rat (12) and human (13) intestinal cDNA libraries. Cells expressing these receptors specifically bind ^{125}I-STa and respond to the toxin with a dramatic increase in their intracellular cGMP content. The STa receptor retains the general structure of the natriuretic peptide receptors A and B (NPR-A, NPR-B) (14, 15). In all three examples, a single transmembrane domain separates the extracellular binding domain from the intracellular region. The latter contains a protein kinase-like domain followed by a COOH-terminal guanylyl cyclase catalytic domain. Intrinsic kinase activity of the kinase homology domain has not yet been demonstrated for any of the receptor-guanylyl cyclases, but this domain has been shown to be required for ligand-dependent signaling through NPR-A (16).

The identification of an STa receptor-guanylyl cyclase does not rule out the existence of other STa receptors linked, or unlinked, to guanylyl cyclase. Cross-linking experiments have identified STa binding proteins of many different sizes (17, 18). Cross-linked proteins with a molecular mass of 120–150 kDa probably correspond to the recently cloned STa receptor-guanylyl cyclase. The identity of the 50–80-kDa cross-linked proteins remains to be determined.

In this paper, we describe the characterization of the STaR in human 293 cells that overexpress the cloned human STaR. We also characterized the STaR in the human colonic T84 cell line that is known to bind STa and respond with an increase in guanylyl cyclase activity (19). These results are compared with published data for STa binding to rat brush border membranes.

MATERIALS AND METHODS

Transfection of human embryonic kidney 293 cells with the full-length cDNA coding for the human STaR was as previously described (13). In these studies, however, individual G418-resistant colonies were isolated and analyzed for STaR expression by ^{125}I-STa$_{6-19}$ binding and guanylyl cyclase stimulation. 293 and T84 cell lines were grown in Dulbecco's modified medium Eagle's medium supplemented with F-12 nutrient mixture, 20 mM Hepes[1] (pH 7.4), and 10% fetal bovine serum. Transfected 293 cell lines were maintained under selection in the presence of 450 μg/ml Geneticin (GIBCO). STa$_{6-19}$ was radioiodinated to a specific activity of 2000 Ci/mmol using Na^{125}I and chloramine T (20). Briefly, 10 μg of STa was incubated on ice with 1 mCi of Na^{125}I to which 10 μg of chloramine T was added. The reaction was left for 30 s, and the iodinated STa$_{6-19}$ was purified by reverse phase HPLC as described (21).

Stable 293 cell lines were washed three times with phosphate-buffered saline (PBS), then harvested using PBS + 1

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[1] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; NPR, natriuretic peptide receptor.

mM EDTA, and resuspended in PBS containing 0.1% bovine serum albumin, 0.02% azide (PBSA buffer). Cells were incubated with 25 pM ^{125}I-STa$_{6-19}$, unless otherwise indicated, in the presence or absence of various concentrations of STa or analogs. Nonspecific binding was determined in the presence of 500 nM STa$_{6-19}$. Incubation was performed under gentle agitation for 2 h at 37 °C. Binding was terminated by filtration through Whatman GF/C filters pretreated with 1% polyethyleneimine. Filters were rinsed three times with 2 ml of ice-cold PBS and counted in a γ counter (Iso-Data 100). Binding of ^{125}I-labeled STa$_{6-19}$ to T84 cells was performed directly on the cells in 12-well tissue culture plates. Each well contained approximately 6×10^5 cells. Binding was performed as described for 293-STaR cells. After incubation, cells were rinsed three times with cold PBS, removed with trypsin for 15 min at 37 °C, and the amount of ^{125}I-STa$_{6-19}$ bound was determined.

Association kinetics of ^{125}I-STa$_{6-19}$ binding were determined at an STa$_{6-19}$ concentration of 25 pM in the presence or absence of 500 nM cold STa$_{6-19}$. Binding was started at different times with the addition of a suspension of 293-STaR cells in a final volume of 0.25 ml and stopped by filtration as described above. For dissociation experiments, a suspension of 293-STaR cells was incubated in the presence of 25 pM labeled STa$_{6-19}$ for 2 h at 37 °C in order to achieve equilibrium. At that time, the cells were centrifuged and rapidly suspended in the same volume of buffer containing 500 nM STa$_{6-19}$. Aliquots (3×0.25 ml) were then removed after various times and immediately filtered. The association rate constant K_1 was determined from the equation: $K_1 = (K_{obs} - K_2)/[L]$, where K_{obs} is the slope of the semi-logarithmic plot of initial association data and $[L]$ is the radioligand concentration. K_2 is the dissociation rate constant and corresponds to the slope of the semi-logarithmic plot of dissociation data.

Peptides were synthesized via solid phase methodology (22) on an ABI model 430 peptide synthesizer using *t*-butoxycarbonyl chemistry. The peptides were cleaved from the resin with hydrogen fluoride and cyclized at a concentration of approximately 0.5 mg/ml via air oxidation at pH 8.0. Crude cyclic peptides were purified by HPLC and analyzed via amino acid analysis and mass spectrometry.

STaR was immunoprecipitated in the presence of protease inhibitors (0.1 mM phenylmethanesulfonyl fluoride, 20 μg/ml aprotinin) as described (23) using polyclonal antibody raised against a fusion protein between the extracellular domain of the STaR and the heavy chain of a human immunoglobulin IgG-γ_1.[2] Immunoprecipitated proteins were solubilized in sample buffer in the presence of dithiothreitol for 5 min at 100 °C and then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% acrylamide gel. The gel was fixed in 10% acetic acid and 10% methanol and enhanced by soaking in Amplify (Amersham Corp.). After drying, the gel was exposed to a storage phosphor imaging plate for 12 h and then developed with a Fuji BAS-2000 Bio-Image Analyzer (Fuji Medical Systems, Stamford, CT).

2×10^5 293-STaR cells were incubated in the presence of 1 nM ^{125}I-STa$_{6-19}$ for 2 h at 37 °C in the presence or absence of unlabeled STa$_{6-19}$in PBSA (pH 7.0). At the end of the incubation, the cells were chemically cross-linked with disuccinimidyl tartrate at a final concentration of 1 mM for 30 min at room temperature. Cells were then centrifuged and washed three times with PBS. The cell pellet was solubilized in sample buffer in the presence of dithiothreitol for 5 min at 100 °C and then analyzed by SDS-PAGE on a 7.5% acrylamide gel.

[2] F. de Sauvage and D. Goeddel, unpublished results.

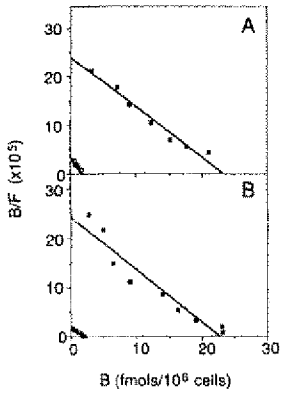

FIG. 1. **Scatchard analysis of ^{125}I-STa$_{6-19}$ binding to 293-STaR cells (■) and T84 cells (□) at 37 °C.** *A*, displacement studies. Cells were incubated with 25 pM ^{125}I-STa$_{6-19}$ in the presence of increasing concentrations of unlabeled STa$_{6-19}$ as indicated. *B*, saturation binding. Cells were incubated with varying concentrations of radiolabeled STa$_{6-19}$ for 2 h. Binding shown here and in Fig. 3 represents specific binding. Nonspecific binding was approximately 5% of total binding.

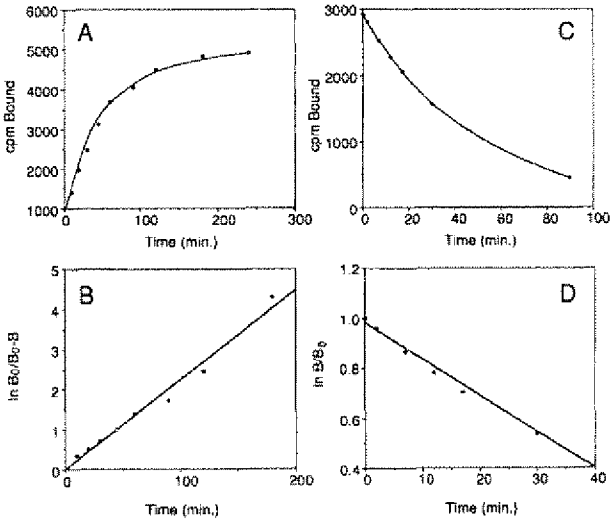

FIG. 2. **Association and dissociation kinetics for binding of ^{125}I-STa$_{6-19}$ to 293-STaR cells.** *A*, association kinetics. Cells and radiolabeled STa were incubated in the presence and absence of excess unlabeled STa$_{6-19}$ at 37 °C for the times indicated. Bound and free counts were separated by vacuum filtration, and the filters were counted. *B*, semi-logarithmic plot of association data. The data are plotted as $\ln B_0/(B_0 - B)$ *versus* association time where B_0 is specific binding at steady state and B is specific binding for the association time shown. *C*, dissociation kinetics. Cells and radiolabeled STa$_{6-19}$ were incubated at 37 °C for 2 h to attain steady-state binding. At that time, the cells were centrifuged and rapidly suspended in the same volume of buffer containing 0.5 μM STa$_{6-19}$. Samples were then incubated for the indicated times and terminated by vacuum filtration and counted. *D*, semi-logarithmic plot of dissociation data. The data are plotted as $\ln B/B_0$ *versus* dissociation time where B_0 is specific binding at steady state and B is specific binding for the dissociation time shown.

Equilibrium binding data were analyzed by Ligand (24) as modified for the IBM personal computer by McPherson (25).

RESULTS AND DISCUSSION

The generation of a G418-resistant pool of human embryonic kidney 293 cells that express recombinant STaR has

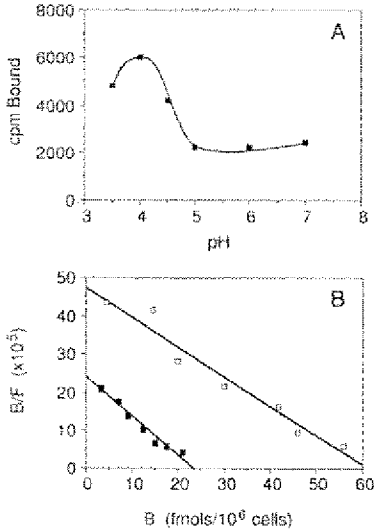

FIG. 3. **STa binding to 293-STaR cells as a function of pH.** *A*, pH profile of ^{125}I-STa$_{6-19}$ binding (25 pM) to 293-STaR cells. *B*, Scatchard analysis of saturation binding to 293-STaR cells at pH 4.0 (□) and pH 7.0 (■). Binding conditions were as described in the legend to Fig. 1.

TABLE I

Displacement of ^{125}I-STa$_{6-19}$ with STa$_{6-19}$ analogues

Inhibition of binding of ^{125}I-STa$_{6-19}$ to 293-STaR cells or T84 cells by unlabeled analogs of STa$_{6-19}$. Relative receptor binding affinities (RA) were calculated as the ratio between the concentration of unlabeled STa$_{6-19}$ giving half-maximal inhibition (K_i) of radiolabeled STa$_{6-19}$ and the concentration of analog giving half-maximal inhibition. BBM, brush border membrane; D-Cys, deoxy-Cys.

Analog	293-StaR		T84		Rat BBM
	K_i	RA	K_i	RA	RA
	nM		*nM*		
STa$_{6-19}$	0.1	1.0	0.1	1.0	1.0[a]
Ala^{6}STa$_{6-19}$	0.5	0.2	0.4	0.25	0.2[a]
Ala^{8}STa$_{6-19}$	5.0	0.02	10	0.01	0.05[b]
Ala^{12}STa$_{6-19}$	5.0	0.02	10	0.01	ND[a,b]
D-Cys^{6}STa$_{6-19}$	0.1	1.0	0.1	1.0	1.0[c]

[a] Ref. 28.
[b] No binding detected.
[c] Ref. 27.

been described (13). The pooled transfected cells expressed a high density of STaR and bound radiolabeled STa while the untransfected control cells showed no binding (13). The same expression system was used to generate individual G418-resistant clones expressing STaR. Twenty clones were isolated and analyzed for STaR expression by ^{125}I-STa$_{6-19}$ binding and guanylyl cyclase stimulation. The clone expressing the highest number of receptors, called 293-STaR, was selected for further characterization. In parallel studies, we have examined the binding of STa to T84 cells, a human colonic cell line that has been shown to specifically bind STa and respond with an increase in guanylyl cyclase activity (19). For these studies, we have used a synthetic peptide (STa$_{6-19}$) encompassing the 13 amino acids necessary for activity as well as the carboxyl-terminal tyrosine (26). This peptide has been shown to be as potent as the full-length 19-amino acid STa peptide in the ability to induce diarrhea in suckling mice (26).

293-STaR cells were incubated with 25 pM ^{125}I-STa$_{6-19}$ in the presence of increasing concentrations of unlabeled

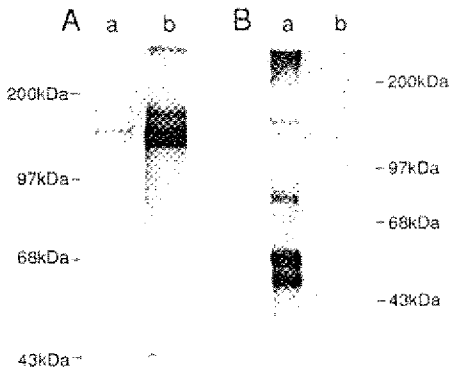

FIG. 4. **SDS-PAGE analysis of STa receptors in 293-STaR cells.** *A*, immunoprecipitation. 100 μg of ^{35}S-labeled T84 (*a*) or 293-STaR (*b*) cells were immunoprecipitated with a polyclonal antibody against the extracellular domain of the STaR and analyzed by SDS-PAGE on a 10% acrylamide gel. *B*, 293-STaR cells were covalently cross-linked with ^{125}I-STa$_{6-19}$ in the absence (*a*) or in the presence (*b*) of 500 nM unlabeled STa$_{6-19}$ and analyzed by SDS-PAGE.

STa$_{6-19}$ for 2 h at 37 °C, conditions which ensure equilibrium binding. Scatchard analysis of the displacement data (Fig. 1*A*) revealed a single binding site with an affinity (K_i) of 97 pM. A similar analysis of ^{125}I-STa$_{6-19}$ binding to human T84 cells (Fig. 1*A*) gave a calculated K_i of 55 pM. This value is not significantly different from that observed for the cloned receptor.

Saturation isotherms were performed with ^{125}I-STa$_{6-19}$ on both 293-STaR and T84 cells. Scatchard analyses of these data (Fig. 1*B*) revealed a single saturable binding component on both cell lines with a K_d of 94 pM with 14,000 sites/cell for the 293-STaR cells and a K_d of 166 pM with 1600 sites/cell for the T84 cells. Agreement between both K_i and K_d indicates that iodination of STa$_{6-19}$ did not significantly alter the binding properties of the toxin. Interestingly, the K_d cited here for STa binding is at least an order of magnitude lower than values previously reported, which range from 2 nM in rat intestine (11) to 12 nM in human T84 cells (19). The higher binding affinity that we obtained in these studies is probably reflective of many different factors including the greater purity of the synthetic peptide relative to the purified toxin preparations or species differences between the human and the rat receptor as suggested by the low similarity between the primary sequences of their extracellular domain (13).

The association of ^{125}I-STa$_{6-19}$ to 293-STaR cells as a function of time is shown in Fig. 2*A*. At 37 °C steady state binding was reached after 2 h. Semi-logarithmic transformation of the data yielded a linear plot, consistent with association to a homogenous population of specific binding sites (Fig. 2*B*). The experimentally observed association rate constant (K_{obs}) for the STaR binding determined from the slope of the plot is 0.022 min^{-1}. An association rate constant, K_1, of 2.4×10^8 M^{-1} min^{-1} was determined.

Dissociation of labeled STa$_{6-19}$ bound to its receptor was initiated by the addition of a large excess of unlabeled STa$_{6-19}$ to a suspension of 293-STaR cells prebound with ^{125}I-STa$_{6-19}$ (Fig. 2*C*). The dissociation rate constant, K_2, determined from the slope of a semi-logarithmic plot of dissociation data (Fig. 2*D*) was 0.016 min^{-1}, and the half-time of dissociation was 43 min. The K_d calculated from the ratio of the kinetic constants K_2/K_1 was 67 pM, which is in very close agreement with the values obtained from steady state binding experiments.

Binding of ^{125}I-STa$_{6-19}$ to 293-STaR cells measured as a function of pH indicated that maximal binding occurred at

pH 4.0 (Fig. 3*A*), as previously reported for T84 cells (19). To determine whether this effect was due to an increase in receptor numbers or in binding affinity, saturation isotherms were performed at pH 4.0 and 7.0. Scatchard analysis of these data (Fig. 3*B*) indicates that the affinity is the same at both pH values but that the number of sites is almost 3-fold higher at pH 4.0 than at pH 7.0 (14,000 sites/cell *versus* 37,000 sites/cell). Chromatography of ^{125}I-STa$_{6-19}$ on a G-25 exclusion column indicated that the ligand migrated at the same position at pH 4.0 and 7.0 (data not shown). Thus, the increased number of binding sites measured is not due to aggregation of labeled STa$_{6-19}$ at low pH. Therefore it is possible that the pH shift results in an unmasking of cryptic receptor sites. The physiologic relevance of the increase in STaR binding sites at pH 4.0 is as yet unknown, but it is tempting to speculate a role for the STaR in the proximal region of the small intestine where acidic conditions prevail during the release of the chyme in the duodenal bulb.

Analogs of the atrial natriuretic peptide have been very useful in helping to identify different subtypes of NPR. It has been shown that the natriuretic peptide clearance receptor, NPR-C, imposes less structural constraints on ligand than does the atrial natriuretic peptide receptor-guanylyl cyclase NPR-A (27). Therefore, five analogs of STa$_{6-18}$ with substitutions at positions potentially important for activity were synthesized and evaluated for their ability to displace ^{125}I-STa$_{6-19}$ binding on both 293-STaR cells and T84 cells. The inhibition constants (K_i) determined for these analogs are shown in Table I. Substitution of either Asn^{12} or Leu^{9} to Ala has a dramatic effect on the ability of the analogs to displace ^{125}I-STa$_{6-19}$ from both cell lines, resulting in an increase in K_i of about 100-fold. Substitution of Glu^{8} to Ala, however, leads only to a 5-fold decrease in affinity. Substitution of Cys^{6} to a D-Cys has previously been shown to generate a long lasting agonist of the STa receptor in the suckling mouse assay (28). The affinity of this analog was the same as STa$_{6-18}$ on both cell lines.

Our results generally correlate with those obtained with rat intestinal membranes (28, 29). The exception was the data obtained with the substitution of Asn^{12} to Ala. In contrast to our data, previous studies with this analog suggested no binding to rat brush border membranes (29). Again, this difference might be attributable to species difference between the rat and the human STaR.

Immunoprecipitation of ^{35}S-labeled 293-STaR and T84 cells with an anti-STaR polyclonal antibody revealed two proteins of approximately 140 and 160 kDa in both cell lines (Fig. 4*A*). Significantly larger amounts of receptor were detected after immunoprecipitation of 293-STaR cells compared with T84 cells. Immunoprecipitation of ^{35}S-labeled control 293 cells did not reveal any specific protein (data not shown).

Receptors present at the cell surface of 293-STaR cells were cross-linked to ^{125}I-STa$_{6-19}$ with disuccinimidyl tartrate. Proteins were separated on SDS-PAGE under reducing conditions and visualized by exposure to an imaging plate. Proteins of 153, 133, 81, 68, 56, and 49 kDa were detected (Fig. 4*B*, *lane A*), the two smallest being the most abundant. All of these bands were completely displaced when the cross-linking was performed in the presence of 500 nM unlabeled STa$_{6-19}$ (Fig. 4*B*, *lane B*). No cross-linked proteins were identified on control 293 cells (data not shown). These lower molecular weight proteins were also detected by immunoprecipitation after overexposure of the gel (data not shown). Preferential cross-linking of ^{125}I-STa to these lower molecular weight proteins with disuccinimidyl suberate has been reported in rat intestinal membranes (17, 18). The 153- and 133-kDa bands detected by cross-linking correlate with the doublet revealed by immunoprecipitation (140 and 160 kDa) and probably correspond to the intact receptor-guanylyl cyclase. However, our results seem to indicate that the smaller molecular weight proteins identified by chemical cross-linking of ^{125}I-STa to rat brush border membranes are not the result of cross-linking to a different receptor but are due to proteolytic processing of the receptor-guanylyl cyclase. Such proteolytic events could account for the earlier identification of the STa binding protein and the guanylyl cyclase activity on separate proteins (10, 11). The results obtained with the 293-STaR cell line suggest that the major STa receptor present on T84 cells and on rat enterocytes corresponds to the cloned STa receptor-guanylyl cyclase (12, 13).

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